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Immune Responses and Protection Obtained by Oral Immunization with Rotavirus VP4 and VP7 DNA Vaccines Encapsulated in Microparticles

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Protective immune responses in mice were obtained after oral immunization with rotavirus DNA vaccines encapsulated in poly(lactide-co-glycolide) (PLG) microparticles. The DNA vaccines used encoded outer capsid proteins VP4 and VP7; proteins that are the basis for rotavirus serotyping and the generation of virus neutralizing antibodies. One dose of vaccine was given to BALB/c mice by oral gavage (75 μg DNA/mouse). Rotavirus-specific serum antibodies and intestinal IgA antibodies were detectable by 6 weeks postimmunization. After challenge with homologous murine rotavirus at 12 weeks postimmunization, fecal rotavirus antigen was reduced significantly in immunized mice compared with controls. Protective immunity also was generated by oral delivery of unencapsulated VP 7 DNA vaccine but to a lesser degree. These results demonstrate that the oral route is effective for generating protective immune responses with rotavirus DNA vaccines targeting neutralization antigens.

INTRODUCTION

Infections with group A rotaviruses cause an estimated 870,000 deaths each year in developing countries (Glass et al., 1996). In the United States, pediatric rotavirus infections are responsible for 55,000–70,000 hospitalizations per year with an estimated cost of more than $1 billion (Glass *et al.,* 1996). Because of the widespread nature of rotavirus disease, development of vaccines is considered key to its control (Bishop, 1993; Glass *et al.,* 1996). The currently available live, attenuated oral rotavirus vaccine is promising (Pérez-Schael *et al.,* 1997), but improved vaccines still are needed, particularly in many developing countries where the need is the greatest (Bishop, 1993; Glass *et al.,* 1996, Keusch and Cash, 1997; Vesikari, 1997) but where the live oral vaccine has been less effective (Lanata *et al.,* 1996; Linhares *et al.,* 1996). Killed rotavirus vaccines and subunit vaccines may be possible (Bishop, 1993), but these types of vaccines do not provide endogenously synthesized proteins, and unless adjuvants are used, generally do not elicit cytotoxic T lymphocyte (CTL) responses that may be important in controlling rotavirus infection. Plasmid DNAs encoding specific viral proteins permit expression of immunizing proteins by host cells that take up inoculated DNA. This results in the presentation of normally processed proteins to the immune system, which is important for raising immune responses against the native forms of proteins (Fynan *et al.,* 1993; Webster *et al.,* 1994). Expression of the immunogen in host cells also results in the immunogen having access to class I major histocompatibility complex presentation, which is necessary for eliciting $CD8^+$ CTL responses.

Rotavirus proteins that elicit neutralizing antibodies are the two outer capsid surface proteins, viral protein (VP) 4 and VP7 (Estes and Cohen, 1989). Before the development of a serotyping scheme for rotaviruses that included VP4, VP7 was the sole basis for rotavirus serotyping. Rotavirus VP7 is the major neutralization antigen of the outer capsid and is also the basis on which the live, attenuated reassortant rotavirus tetravalent vaccine is designed (Pérez-Schael *et al.,* 1997). Previously we found that VP7 and VP4 DNA vaccines given by gene gun gave protective immunity (Herrmann *et al.,* 1996a,b; Chen *et al.,* 1997). One correlate of protection against rotavirus infection is production of rotavirus-specific intestinal IgA *in vivo* (Feng *et al.,* 1994). Thus targeting of rotaviruses to the gut-associated lymphoid tissue to induce intestinal IgA may be important in the development of rotavirus vaccines. Using a method for encapsulation of DNA in poly(lactide-co-glycolide) (PLG) microparticles (Jones *et al.*, 1997a,b), we demonstrated (Chen *et al.,* 1998) protective immunity to rotaviruses with a PLG-encapsulated DNA vaccine encoding the rotavirus antigen VP6. Rota-

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TABLE 1

Rotavirus-Specific Serum ELISA Antibodies in BALB/c Mice after Oral Inoculation of PLG-Encapsulated Murine VP7 or VP4+VP7 DNA Vaccines, or Unencapsulated Murine VP7 DNA Vaccine

Weeks postimmunization	DNA vaccine			
	PLG-VP7	PLG-VP4+VP7	VP7 unencapsulated	Plasmid control
0	< 2 (0/5)	< 2 (0/6)	< 2 (0/6)	< 2 (0/5)
6	2.3 ± 0.00 (5/5)	2.54 ± 0.13 (6/6)	< 2 (0/6)	< 2 (0/5)
8	2.54 ± 0.25 (5/5)	2.70 ± 0.16 (6/6)	2.3 ± 0.0 (3/6)	< 2 (0/5)
10	< 2 (0/5)	2.85 ± 0.12 (6/6)	< 2 (0/6)	< 2 (0/5)

Note. $1/\log_{10}$ ELISA titer, mean values ± SD (responders/number tested). Mean values ± SD are for responders.

virus VP6 is located on the first inner capsid and is not exposed unless the virion is disrupted. It is antigenically conserved among group A rotaviruses but, unlike antibodies to VP4 and VP7, does not elicit antibodies that neutralize rotavirus *in vitro*. The results that we obtained with a PLG-encapsulated rotavirus VP6 DNA were the first to demonstrate protection against an infectious agent elicited after oral administration of a DNA vaccine.

In this study, we examined the efficacy of a PLG-encapsulated rotavirus VP7 DNA vaccine to induce serum and mucosal antibody responses in adult BALB/c mice and to protect against infection after rotavirus challenge. We also tested a combination of VP7 and VP4 DNA vaccines encapsulated in the same PLG preparation to determine whether the VP4 DNA vaccine would enhance protective immune responses. Finally for comparison with PLG-encapsulated DNA vaccines, we tested for induction of protective immune responses to an unencapsulated ("naked") VP7 DNA vaccine given orally in saline.

RESULTS

Serum antibody production

Inoculated mice were examined for serum total antibodies (IgG, IgM, and IgA) every 2 weeks for 12 weeks. Serum antibody titers obtained by the PLG-encapsulated VP7 and VP4+VP7 DNA vaccines and the unencapsulated VP7 DNA vaccine are given in Table 1. A 1:100 dilution was the lowest one tested. At the one time point where antibodies were generated in three of six mice by the unencapsulated VP7 DNA vaccine, the titer was not significantly different from the titer obtained with the PLG-encapsulated VP7 DNA vaccine at that time point. Serum antibodies in the mice that had received live rotavirus (used as a positive control) had mean 1/enzyme-linked immunosorbent assay (ELISA) titers ($\log_{10}$) of 4.1 at Week 10 p.i.

Detection of rotavirus-specific serum IgA was detected in immunized mice at the times shown in Table 2. Not all mice in each group responded to the DNA vaccines. The most consistent responses occurred in mice that had been immunized with the PLG-encapsulated VP4+VP7 DNA vaccine.

Protection against rotavirus challenge

Immunized mice were challenged with 100 ID_{50} epizootic diarrhea of infant mice (EDIM) rotavirus at 12 weeks p.i. The results, expressed as ELISA values for detection of rotavirus antigen shed in stools, are shown in Fig. 1. Significant reductions in virus antigen shed were noted on Days 3–5 for mice that had been immunized with PLG-encapsulated VP7 or VP4+VP7 DNA vaccines or with EDIM rotavirus. In the same set of experiments, mice were immunized with unencapsulated VP7 DNA vaccine to determine the efficacy of encapsu-

TABLE 2

Rotavirus-Specific Serum IgA Antibodies in BALB/c Mice after Oral Inoculation of PLG-Encapsulated Murine VP4 or VP4+VP7 DNA Vaccines, or Unencapsulated Murine VP7 DNA Vaccine

Weeks postimmunization	EDIM virus	DNA vaccine			
		PLG-VP4+VP7	PLG-VP7	VP7 unencapsulated	PLG-plasmid control
0	< 0.10 (4/4)	< 0.10 (0/6)	< 0.10 (0/5)	< 0.10 (0/6)	< 0.10 (0/5)
6	0.43 ± 0.04 (4/4)	0.20 ± 0.001 (3/6)	< 0.10 (0/5)	< 0.10 (0/6)	< 0.10 (0/5)
8	Not tested	0.20 ± 0.01 (4/6)	< 0.10 (0/5)	0.23 ± 0.07 (3/6)	< 0.10 (0/5)
10	0.38 ± 0.05 (4/4)	0.24 ± 0.03 (6/6)	0.23 ± 0.03 (2/5)	0.20 (1/6)	< 0.10 (0/5)

Note. Serum IgA (μg/ml), mean values ± SD (responders/number tested). Mean values ± SD are for responders.

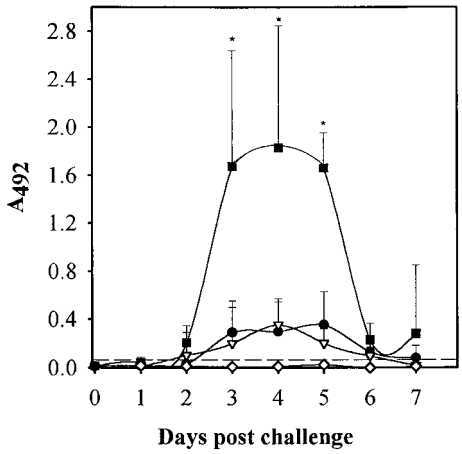

FIG. 1. Protection against EDIM rotavirus challenge in BALB/c mice orally immunized with PLG-encapsulated VP7 and PLG-encapsulated VP4+VP7 DNA vaccines. Virus shedding in feces was determined by an ELISA for detecting rotavirus antigen and the results reported as A_{492} values ± SD. A positive test is one in which the A_{492} value is ≥0.1. There were significant differences ($P < 0.0002$, analysis of variance) in viral shedding between the mice receiving the PLG-encapsulated plasmids encoding VP7 or VP4+VP7 and the plasmid control on the days indicated by an asterisk. ◇, mice inoculated with EDIM virus, $n = 4$; ▽, mice inoculated with PLG-VP7 DNA vaccine, $n = 5$; ●, mice inoculated with PLG-VP4+VP7 DNA vaccine, $n = 6$; ■, mice inoculated with control plasmid DNA, $n = 5$.

lation on generation of protective immunity. Mice immunized with unencapsulated VP7 DNA vaccine showed significant protection only on Day 5 postchallenge (Fig. 2). Data points for the plasmid control shown in Fig. 2 were the same as those in Fig 1. Compared with the unencapsulated DNA vaccine, there was significantly greater reduction of antigen shedding in mice receiving the PLG-encapsulated DNA vaccine on Days 4 and 5.

Rotavirus-specific antibodies in stools

Mice were examined for intestinal rotavirus-specific IgA antibodies before and after virus challenge. IgA was detected in feces of mice that had been immunized with the PLG-encapsulated VP4+VP7 DNA vaccine or EDIM rotavirus at 6, 8, 10, and 12 weeks postimmunization (data not shown). For the DNA vaccine, this suggested that rotavirus antigen was expressed and a mucosal antibody response had been induced. No rotavirus-specific IgA was detected in stools of mice immunized with PLG-encapsulated VP7 DNA vaccine, unencapsulated VP7 DNA vaccine, or the PLG-encapsulated control plasmid DNA.

After virus challenge, in addition to testing for rotavirus-specific IgA antibodies in feces, we tested for possible enhancement of intestinal rotavirus IgG antibodies with an IgG-specific ELISA. There were significant differences ($P < 0.01$) in fecal IgA and IgG values between the mice receiving the plasmid DNA control and the mice that had received the PLG-encapsulated VP4+VP7 DNA vaccine or live EDIM rotavirus (Figs. 3A and 3B) ($P < 0.01$ for EDIM rotavirus immunized mice on Days 1–7 for IgA and IgG; $P < 0.01$ for VP4+VP7 DNA vaccine on Days 3 and 5–7 for IgA and Days 4–7 for IgG). There was no significant enhancement of rotavirus-specific IgA or IgG in feces of mice that had received the PLG-encapsulated VP7 DNA vaccine or unencapsulated VP7 DNA vaccine.

DISCUSSION

Protective immune responses against rotavirus infections have been correlated with production of rotavirus-specific fecal IgA *in vivo* in human and porcine studies as well as in mice (Matson e*t al.,* 1993; Feng e*t al.,* 1994; Ward, 1996; Yuan e*t al.,* 1996; Chen e*t al.,* 1998). Thus induction of intestinal IgA may be an important correlate in the development of rotavirus vaccines. Serum IgA has also been correlated with protection in mice (Feng e*t al.,* 1994; McNeal *et al.,* 1994). In mice immunized by gene gun with VP4, VP6, or VP7 DNA vaccines (Herrmann *et al.,* 1996a,b; Chen e*t al.,* 1997) and in mice orally immunized with a PLG-encapsulated VP6 DNA vaccine (Chen e*t al.,* 1998), we had seen enhanced intestinal IgA responses after they had been rotavirus challenged. With the PLG-encapsulated VP6 DNA vaccine, we also had detected rotavirus-specific intestinal IgA responses before the mice were challenged. Here we found that oral immunization of the PLG-encapsulated VP4+VP7 DNA vaccine also induced rotavirus-specific intestinal IgA responses before challenge and enhanced intestinal IgA responses to rotavirus after virus challenge. Serum IgA was detected in mice after immunization with each of the DNA vaccines tested, including the unencapsulated VP7 DNA vaccine.

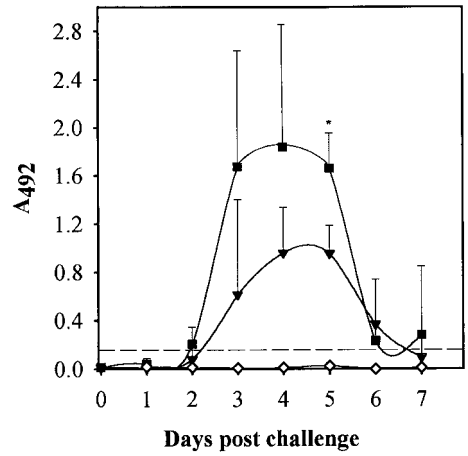

FIG. 2. Protection against EDIM rotavirus challenge in BALB/c mice orally immunized with unencapsulated (naked) DNA vaccine. Virus shedding in feces was determined by an ELISA for detecting rotavirus antigen and the results reported as A_{492} values ± SD. A positive test is one in which the A_{492} value is ≥0.1. There were significant differences ($P < 0.0002$, analysis of variance) in viral shedding between the mice receiving the plasmid encoding VP7 and the plasmid control on the day indicated by an asterisk. ◇, mice inoculated with EDIM virus, $n = 4$; ▼, mice inoculated with unencapsulated VP7 DNA vaccine, $n = 6$; ■, mice inoculated with control plasmid DNA, $n = 5$.

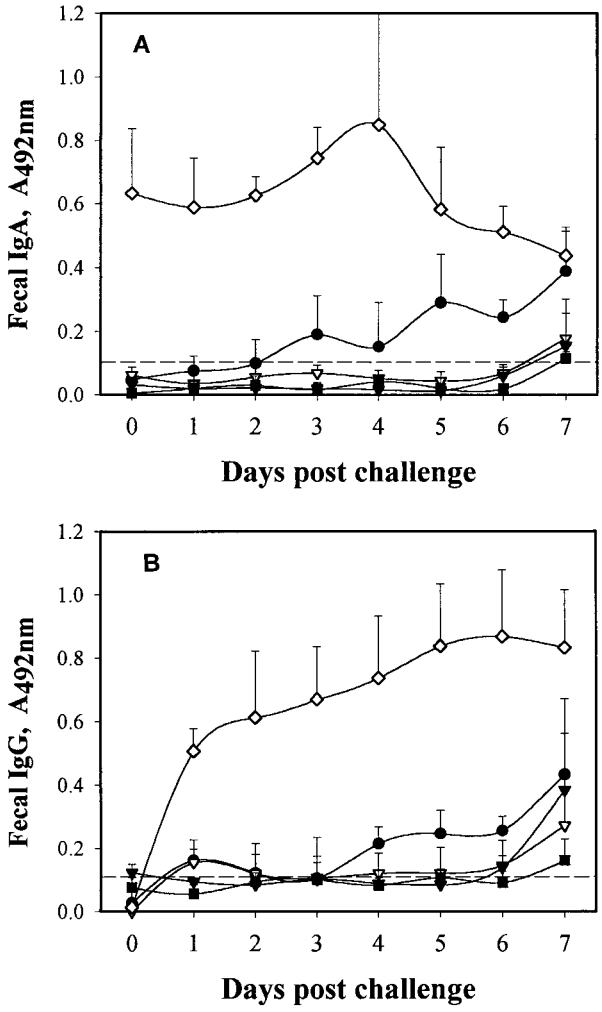

FIG. 3. (A) and (B) ELISA for rotavirus specific IgA (A) or IgG (B) in stool suspensions from mice that had been orally inoculated with EDIM virus or DNA vaccines and challenged with EDIM rotavirus. ◇, mice inoculated with EDIM virus, $n = 4$; ▽, PLG-encapsulated VP7 DNA vaccine, $n = 5$; ●, PLG-encapsulated VP4+VP7 DNA vaccine, $n = 6$; ▼, unencapsulated (naked) VP7 DNA vaccine, $n = 6$; ■, PLG-control plasmid DNA, $n = 5$. The stools were diluted 1:80 (w/v) in PBS. Results are expressed as A_{492} values ± SD. A_{492} values >0.1 are considered positive for IgA and IgG.

We concentrated on the DNA vaccine encoding rotavirus VP7 because VP7 is the major neutralization antigen of the outer capsid and has been the basis for the design of the live, attenuated reassortant tetravalent rotavirus vaccine (Pérez-Schael *et al.,* 1997). For the vaccine that contains the plasmids encoding VP4 and VP7 encapsulated together, we are presuming that some, if not most of the DNAs, will be expressed in the same cell, but this remains to be shown. There were higher serum antibody titers obtained with the PLG-encapsulated VP4+VP7 DNA vaccine than with the PLG-encapsulated VP7 DNA vaccine and enhancement of fecal antibody titers after virus challenge, but there did not appear to be any difference in the protection obtained. It remains to be determined if the enhanced antibody responses seen were due solely to the addition of VP4 or were obtained because the two plasmids were encapsulated in the same microparticles. The contribution of antibodies raised by each of the DNA vaccines to the antibody titers measured was not established. In future studies we hope to have assays available for detecting antibodies specific for VP4 and VP7. We also will need to determine how well a PLG-encapsulated VP4 DNA vaccine will protect mice when given alone.

As we found previously with the PLG-encapsulated VP6 DNA vaccine (Chen *et al.,* 1998), more DNA is required when encapsulated and given orally than is required for gene gun delivery (Herrmann *et al.,* 1996a,b; Chen *et al.,* 1997) (75 μg compared with two doses of 2.5 μg given by gene gun). However, only one inoculation is required for PLG-encapsulated DNA vaccines given orally, whereas gene-gun immunization for rotaviruses has required multiple inoculations (Herrmann *et al.,* 1996a,b; Chen *et al.,* 1997; Choi *et al.,* 1998). The relative effectiveness of the two methods has not been directly compared in one experiment, but we expect to do this in future studies.

We have not determined the cell or cells targeted by the encapsulated DNA, but it is likely that similar cells are involved as those that have been shown to be involved in the uptake of PLG microparticles encapsulating antigens (Eldridge *et al.,* 1989; Desai *et al.,* 1996; O'Hagen, 1996), as we previously discussed in detail (Chen *et al.,* 1998). PLG microparticles are not selectively targeted to M cells, but nonspecific binding to M cells and subsequent transcytosis has been shown in rabbits (Jepsom *et al.,* 1993, 1996).

In addition to binding of PLG by M cells, it is assumed that encapsulation protects the DNA from degradation by nucleases. It had been shown in other studies that naked DNA encoding luciferase did induce immune responses, but PLG encapsulation enhanced the responses (Jones *et al.,* 1997a), and CTL responses to naked DNA encoding measles antigen given orally generated CTL responses to that antigen (Etchart *et al.,* 1996). We obtained partial protection after oral administration of naked VP7 DNA vaccine, but the consistency of the antibody responses measured and the protection obtained were greater with the PLG-encapsulated VP7 DNA vaccine. We will be testing unencapsulated VP4 and VP4+VP7 DNA vaccines in future studies.

Adult mice (mice older than 2 weeks) do not develop diarrhea after rotavirus infection. Because of this, protective immunity in the adult mice used was measured by reduction of rotavirus antigen shed after challenge. However, because rotavirus infection can occur in the absence of disease, protection against rotavirus infection may be as stringent or a more stringent measure of protection than protection from disease (O'Neal *et al.,* 1997).

It has been suggested that DNA might make a third generation of rotavirus vaccines (Vesikari, 1997) and may provide more effective rotavirus vaccines in the future.

DNA vaccines encapsulated in PLG microparticles combine the ease of administration by the oral route with targeting of mucosal tissues. The results that we obtained extend the results we previously obtained with a PLG-encapsulated rotavirus VP6 DNA vaccine (Chen *et al.*, 1998) and demonstrate the potential for development of PLG-encapsulated rotavirus DNA vaccines based on neutralization antigens.

MATERIALS AND METHODS

Virus and mice

Epizootic diarrhea of infant mice (EDIM) rotavirus strain EW (P10[16], G3) was used for preparation of cDNA encoding VP4 and VP7 and for virus challenge of mice. The virus challenge stock was prepared by passaging virus from intestinal homogenates of EDIM rotavirus-infected infant mice in adult mice. Virus for challenge was stool samples diluted in saline. The ID_{50} of the stock virus was the 50% shedding dose as determined by detection of rotavirus antigen shed in feces of infected mice. The mice used for vaccine studies were obtained from rotavirus-free colonies (Taconic, Germantown, NY) at 6–8 weeks of age and were housed in plastic microisolater cages before and after immunization. The model developed by Ward e*t al.* (1990) for BALB/c mice was used to measure protective immunity. In this model, the endpoint is infection rather than illness, because illness generally is limited to infant mice aged ≤15 days. The adult mouse (≥6 weeks) becomes infected and sheds virus in feces for ~1 week postinfection. Protection after virus challenge was defined as significant reduction of rotavirus antigen shed in feces during a 7-day period.

DNA vaccines

The plasmids encoding rotavirus VP4 or VP7 were prepared by insertion of murine rotavirus VP4 or VP7 into the pCMV intron A TPA expression vector provided to us by Dr. J. Mullins, University of Washington (plasmid JW4303) (Yasatumi e*t al.,* 1996). This vector uses sequences from the CMV immediate early promoter to drive transcription and sequences from bovine growth hormone genes to provide polyadenylation signals. To prepare VP4 (GenBank Accession No. MRU08429) DNA vaccine by blunt end ligation and VP7 (GenBank Accession No. MRU08430) DNA vaccine by cohesive end ligation, the TPA leader sequence was removed by treatment with restriction endonucleases *Hin*dIII and *Bam*HI. For insertion of VP7, the *Hin*dIII site was changed to a *Bam*HI site. Newly constructed plasmids in the correct orientation were identified by restriction endonuclease digestion. Expression of rotavirus VP4 or VP7 in transfected COS cells was confirmed by indirect immunofluorescent staining with monoclonal antibodies to VP4 or VP7. The control DNA vaccine was plasmid JW4303 without the viral cDNA insert.

Encapsulation of DNA vaccines

Plasmid DNAs were encapsulated in poly (lactide-co-glycolide) (PLG) microparticles by the solvent extraction technique as previously described (Jones e*t al.,* 1996, 1997a,b). In brief, the DNA was emulsified with PLG dissolved in dichloromethane, and this water-in-oil emulsion was emulsified with aqueous polyvinyl alcohol (an emulsion stabilizer) to form a (water-in-oil)-in-water double emulsion. This double emulsion was added to a large quantity of water to dissipate the dichloromethane, which resulted in the microdroplets hardening to form microparticles. The microparticles were harvested by centrifugation, washed several times to remove the polyvinyl alcohol and residual solvent, and lyophilized. PLG encapsulation of the VP4+VP7 DNA vaccine was prepared by mixing the two plasmids in equal proportions before encapsulation. The microparticles containing DNA had a mean diameter of 0.5 μm. To test for DNA content, the microparticles were dissolved in 0.1 M NaOH at 100°C for 10 min. The A_{260} was measured and the amount of DNA calculated from a standard curve. Incorporation of DNA into microparticles was 1.52 μg DNA per milligram PLG for the VP7 DNA vaccine, 2.1 μg DNA per milligram PLG for the VP4+VP7 DNA vaccine, and 1.75 μg DNA per milligram PLG for the plasmid control.

Immunization of mice

BALB/c mice were inoculated orally (by gavage) with PLG-encapsulated plasmid DNA encoding murine rotavirus VP7, VP4 + VP7, or control plasmid DNA (n = 6 mice for each inoculation). The mice were fasted 4–5 h before inoculation. The microparticles were suspended in a solution of 0.1 M sodium bicarbonate in distilled water, pH 8.5, and given at 0.5 ml per mouse. The DNA dose administered was ~75 μg DNA per mouse. In one set of mice (n = 6), unencapsulated VP7 DNA vaccine suspended in saline was inoculated orally (by gavage) at 75 μg DNA per mouse. Another group of mice was immunized with live EDIM rotavirus 12 weeks before challenge for use as a positive control.

Antigen and antibody tests

For monitoring viral antigen shedding in mouse feces, we used a commercial monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) in microtiter plates (Rotaclone, Meridian Diagnostics, Cincinnati, OH) as described by the manufacturer. For evaluating rotavirus-specific serum antibody responses, an indirect ELISA for total antibody (IgG, IgM, and IgA) (Feng e*t al.,* 1994; Herrmann e*t al.,* 1996a,b; Chen e*t al.,* 1997), was used with EDIM rotavirus-coated wells. Peroxidase-labeled anti IgA (goat anti-mouse IgA, Southern Biotechnology Associates, Inc., Birmingham, AL) was used in the ELISA to determine rotavirus-specific serum IgA levels.

Intestinal IgA and IgG antibodies to EDIM virus were determined by use of IgA- or IgG-specific peroxidase-labeled antiglobulin in an indirect ELISA (Feng e*t al.,* 1994; Herrmann e*t al.,* 1996a,b; Chen e*t al.,* 1997). Five percent (wt/vol) stool suspensions in 0.01 M phosphate buffered saline, pH 7.1, were further diluted 1:4 (final dilution of 1:80) for assay of fecal IgA.

Statistical analyses

Statistical analyses were performed using a nonparametric Wilcoxon 2-sample test for ranked data and analysis of variance and the Student-Newman–Keuls test for multiple comparison of the differences among experimental groups.

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